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Okuläre Toxoplasmose-Antikörper in Kammerwasser und Serum

Zusammenfassung

Hintergrund. Die Diagnose der okulären Toxoplasmose basiert auf der ophthalmologischen Untersuchung. Jedoch kann die Diagnosefindung durch eine alleinige klinische Beurteilung Schwierigkeiten bereiten. Ziel unserer Studie war es, den diagnostischen Wert der Kammerwasser- und Serumuntersuchung bei okulärer Toxoplasmose zu bestimmen.
Methoden. Antikörper gegen Toxoplasma gondii wurden bei 50 Patienten mit klinischem Verdacht auf eine Toxoplasmose sowie bei 25 Patienten mit einer Uveitis posterior oder Panuveitis (Kontrollgruppe) im Kammerwasser sowie im Serum bestimmt.
Ergebnisse. Bei 49 Patienten mit Verdacht auf eine Toxoplasmose-Retinochoroiditis konnte eine intraokuläre Antikörpersynthese nachgewiesen werden. 2 Patienten der Kontrollgruppe zeigten eine lokale Antikörperproduktion. 49 von 50 Patienten mit okulärer Toxoplasmose hatten einen erhöhten Serum-IgG-Antikörper-Titer, waren jedoch bis auf 3 Patienten negativ für IgM. Das Serum-IgG gegen Toxoplasmose war in der Kontrollgruppe bei 21 Patienten erhöht, aber negativ für IgM.
Diskussion. Der lokale Antikörpernachweis ist eine zuverlässige Methode zur Diagnosestellung einer Toxoplasmose-Retinochoroiditis. Die Serumanalyse ist von geringerer Bedeutung.

Schlüsselwörter

Toxoplasmose · Kammerwasser · Serologie · Retinochoroiditis

Die okuläre Toxoplasmose ist, großen epidemiologischen Untersuchungen zufolge, die häufigste Ursache der Uveitis posterior [9, 16, 20]. Ihr Anteil an den hinteren Uveitiden beträgt in unserem Patientenkollektiv etwa 30%. Sind charakteristische Zeichen der Toxoplasmose-Retinochoroiditis vorhanden, ist die Diagnosestellung für den Augenarzt oft klinisch möglich. Hierbei zeigt sich in der Regel ein gelblich-weißer Herd mit flauschigen, unscharfen Rändern und evtl. angrenzender Netzhautnarbe. Jedoch sind untypische Manifestationen mit anderen klinischen Erscheinungsformen nicht selten. Atypische Verläufe können sich bspw. als retinale Vaskulitis oder als Erstmanifestation bei älteren Patienten präsentieren.

Andererseits können Erkrankungen wie Syphilis, Tuberkulose, Herpes-simplex-Virus- oder Zytomegalie-Virus-Infektionen eine okuläre Toxoplasmose vortäuschen. Von Interesse ist es darüber hinaus, einen Anhalt über die Aktivität der Erkrankung zu gewinnen bzw. eine sichere Diagnosestellung bei rezidivierenden Verläufen mit zum Teil schwerer Visusminderung zu finden, um die Indikation für eine längerfristige Therapie stellen zu können. Bei Patienten, bei denen keine sichere Diagnose gestellt werden kann, ist eine unterstützende Untersuchungsmethode wünschenswert. Die zwei wichtigsten Laboruntersuchungen sind:

1. Die Bestimmung von Antikörpern gegen Toxoplasmose im Serum, deren diagnostischer Wert aufgrund des starken Durchseuchungsgrades unzuverlässig ist [10].
2. Die Kammerwasseruntersuchung, die schon vor ca. 40 Jahren von Goldmann u. Witmer [7, 22] empfohlen wurde, jedoch nicht als Routinemethode angewendet wird.

Ziel unserer Untersuchung war es daher, die diagnostische Wertigkeit der Serologie und der Kammerwasseranalyse bei Toxoplasma-gondii-Infektionen des Auges zu bestimmen.

Material und Methoden

Wir werteten in einer retrospektiven Studie die Ergebnisse der Serum- und Kammerwasseruntersuchungen von 50 Patienten mit Toxoplasmose aus, die von 1996 bis 2000 in der Universitäts-Augenklinik, Charité Berlin gesehen wurden. Es handelte sich hierbei um 28 Frauen und 22 Männer im Alter zwischen 8 und 71 Jahren. Die Diagnose wurde durch eine erfolgreiche Therapie mit Clindamycin 4×300 mg p.o. bestätigt. Zum Ausschluss anderer Ursachen wurden folgende Parameter bestimmt: HSV-, VZV-, EBV-, CMV-, Lues-, Borrelien-Serologie, ANCA, ANA und Angioconverting-Enzym.

Als Kontrollgruppe wurden insgesamt 25 Patienten konsekutiv in die Un-

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Ocular toxoplasmosis antibodies in aqueous humor and serum

Abstract

Background. The diagnosis of ocular toxoplasmosis is mainly based on ophthalmological examination but might be difficult to establish in some cases. The purpose of our study was to evaluate the value of aqueous humor and serum analysis in ocular toxoplasmosis.
Patients and methods. We analyzed the avidity of toxoplasma-specific IgG in aqueous humor and serum samples from 50 patients with toxoplasmic retinochoroiditis, with 25 patients with uveitis posterior or panuveitis serving as controls.
Results. Specific intraocular antibody synthesis could be confirmed in 49 patients (98%). In two patients (8%) of the control group, antibody synthesis was detected (false positive). Forty-nine patients with diagnoses of ocular toxoplasmosis were positive for serum anti-*T. gondii* IgG, but only three patients had increased IgM levels.
Conclusions. Analysis of local antibody production is a reliable method for confirming or excluding a suspected clinical diagnosis of toxoplasma retinochoroiditis. The determination of toxoplasma antibodies in the patients' serum is of limited value.

Keywords

Toxoplasmosis · Serology · Aqueous humor · Retinochoroiditis

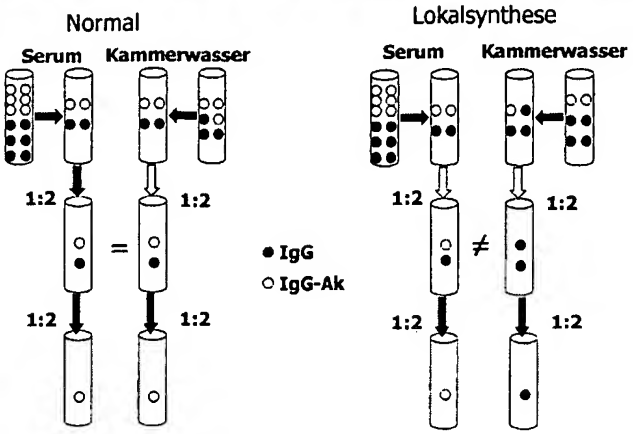

Abb. 1 ▲ **Schematische Darstellung der Kammerwasseranalyse. *Normal*: Keine lokale Antikörpersynthese findet sich, wenn der entsprechende IgG-Gehalt im Kammerwasser kleiner oder gleich dem im Serum ist. *Lokalsynthese*: Der entsprechende IgG-Gehalt ist im Kammerwasser höher als im Serum**

tersuchung eingeschlossen. Dabei handelte es sich um 19 Patienten mit einer Uveitis posterior (12×unklare Diagnose, 1×HSV-, 2×VZV-Retinitis, 1×Toxocara, 3×Vaskulitis) und 6 Patienten mit einer Panuveitis (2×Borreliose, 1×HSV, 1×VZV, 2 unklarer Genese). Nach einer Kammerwasserpunktion erfolgt in unserer Klinik, bei ausreichendem Untersuchungsmaterial, routinemäßig eine Bestimmung von Antikörpern gegen HSV, VZV, EBV und Toxoplasmose.

Die Vorderkammerpunktion erfolgte unter sterilen Bedingungen in Tropfanästhesie mit einer 30G-Kanüle. Über eine mit der Kanüle verbundene Tuberkulinspritze wurden etwa 100–300 µl Kammerwasser aspiriert. Gleichzeitig erfolgte die Entnahme von 5 ml Serum.

Die Kammerwasserproben wurden mit einem, von uns bereits früher beschriebenen, modifizierten Micro-ELISA-Test (Behring, Marburg) auf eine intraokuläre Antikörpersynthese gegen Toxoplasma gondii untersucht [8]. Im Gegensatz zu den bisher üblichen Verfahren, die mit festen Ausgangsverdünnungen arbeiten, werden Kammerwasser und Serum auf einen standardisierten IgG-Gehalt (1 mg/dl) verdünnt. Dies hat den Vorteil, dass bei hoher IgG-Konzentration sehr wenig Probenmaterial eingesetzt werden muss. In weiteren Verdünnungsreihen wird jeweils spezifisch IgG gegen Toxoplasma gondii bestimmt, wobei sich eine lokale Antikörperproduktion dadurch darstellt, dass spezifisches IgG in höherer Verdünnung im Kammerwasser als im Serum nachweisbar ist (Abb. 1).

In einer zweiten Serumprobe erfolgte unabhängig von der oben beschriebenen Kammerwasseranalyse mittels ELISA-Verfahren (VIDAS, BIO-MERIOX) die Toxoplasma-gondii-IgM und -IgG-Antikörperbestimmung in der mikrobiologischen Abteilung der Universitätsklinik Charité.

Ergebnisse

Serumuntersuchung

Die serologische Untersuchung zeigte für die Patienten mit einer Toxoplasmose-Retinochoroiditis bei nur 3 der 50 (6%) Patienten einen Anstieg des IgM-Antikörpertiters gegen Toxoplasma gondii. 49 von 50 (98%) Patienten wiesen einen erhöhten Titer für IgG-Antikörper auf (Tabelle 1). Im Vergleich dazu stellten sich für die serologische Untersuchung in der Kontrollgruppe kaum Unterschiede dar (Tabelle 2). Bei 49 der Patienten der Toxoplasmose-Gruppe (98%) und 21 der Kon-

Tabelle 1
Ergebnisse der Kammerwasseranalyse bei Patienten mit Toxoplasma-gondii-Infektion des Auges

	Kammerwasser Analyse	Serologie IgM	Serologie IgG
Positiv	49 (98%)	3 (6%)	49 (98%)
Negativ	1 (2%)	47 (94%)	1 (2%)

Tabelle 2
Ergebnisse der Kammerwasseranalyse bei Patienten der Kontrollgruppe

	Kammerwasser Analyse	Serologie IgM	IgG
Positiv	2 (8%)	0	21 (84%)
Negativ	23 (92%)	25 (100%)	4 (16%)

trollgruppe (84%) war das IgG positiv. Bei 94% bzw. 100% war das IgM negativ. Die Angaben einer hohen Durchseuchung bestätigten sich auch bei unseren Kontrollpatienten, die einen positiven IgG-Antikörpernachweis in 84% aufwiesen.

Damit ergibt sich für die serologische Diagnostik für IgM zwar eine geringe Sensitivität (6%), jedoch eine hohe Spezifität (100%). Für IgG ergibt sich umgekehrt eine hohe Sensitivität (98%) bei einer geringen Spezifität (16%).

Kammerwasseranalyse

Die Ergebnisse der Kammerwasseruntersuchung belegen eine hohe Sensitivität des intraokulären Antikörpernachweises. Bei 98% der Patienten mit einer Toxoplasmose (49 von 50) wurde eine lokale Antikörperproduktion gegen Toxoplasma gondii nachgewiesen (Tabelle1). Bei einem Patienten beobachteten wir ein negatives Ergebnis. Von den 25 Augen mit einer Uveitis nicht-toxoplasmotischer Genese beobachteten wir bei 2 Patienten ein positives Resultat. Bei 23 der 25 Patienten war das Ergebnis negativ (Tabelle2).

Somit ergibt sich für die Kammerwasseruntersuchung mit 98% eine hohe Sensitivität bei gleichzeitig hoher Spezifität (92%).

Diskussion

Bei der Diagnose einer okulären Toxoplasmose steht das klinische Bild der Erkrankung im Mittelpunkt. Dennoch werden immer wieder Verläufe beobachtet, die nicht eindeutig sind und Ursache schwerer Visusminderungen und irreversibler absoluter Skotome sein können (Abb.2, 3). Unsere Untersuchung zeigt, dass die Kammerwasseruntersuchung, bei der Diagnose einer intraokulären Toxoplasmose-Infektion, eine diagnostische Maßnahme mit hohem Aussagewert ist. Die Sensitivität lag für die Kammerwasseranalyse bei 98% und die Spezifität bei 92%. Dabei verwendeten wir eine modifizierte Mikro-ELISA-Methode, [8, 14] um eine spezifische lokale Antikörperproduktion nachzuweisen. Diese zeigte in früheren Untersuchungen auch bei intraokulären Entzündungen anderer Genese eine hohe Sensitivität und Spezifität [14] und hat den Vorteil, bei geringem Probevolumen eine Bestimmung mehrerer Antikörper zu gestatten.

Bei 2 Patienten der Kontrollgruppe wurde eine intraokuläre Antikörpersynthese beobachtet, die wir als falsch-positives Resultat ansahen. Dies stimmt mit früheren Beobachtungen überein und ist möglicherweise durch eine polyklonale Aktivierung von B-Lymphozyten erklärt, welche eine stärkere intraokuläre Antikörperproduktion verursachen kann [3, 5]. Bei einem dieser beiden Patienten war die Diagnose einer Fuchs-Heterochromie-Zyklitis gestellt worden. Zuvor ist eine Assoziation zwischen der Fuchs-Heterochromie-Zyklitis und Toxoplasma gondii beschrieben worden. Toxoplasmen wurden als mögliche Erreger für diese Form der Iridozyklitis diskutiert [4]. Wir schließen uns jedoch der Untersuchung von Hey an, der einen Zusammenhang ausschloss [13].

Es zeigte sich bei einem Patienten ein falsch-negatives Ergebnis für die Kammerwasseranalyse. Eine mögliche Erklärung hierfür ist, dass die Kammerwasseranalyse nur bedingt für Patienten anwendbar ist, die an einer schweren Blut-Retina-Schrankenstörung leiden. Oder aber der Zeitpunkt der Punktion liegt bei einer frischen Infektion vor der Ausbildung einer humoralen Immunantwort, so dass ein falsch-negatives Ergebnis resultiert. Hier kann evtl. die PCR-Analyse durch den direkten Nachweis der Erreger-DNA überlegen sein. Vergleicht man diese mit unserer Methode der Antikörperbestimmung, lassen sich für beide Verfahren Vor- und Nachteile zeigen. Wichtig scheint hierbei das Zeitintervall zwischen Infektion und der Punktion zu sein [17]. Während in der Frühphase der Infektion wahr-

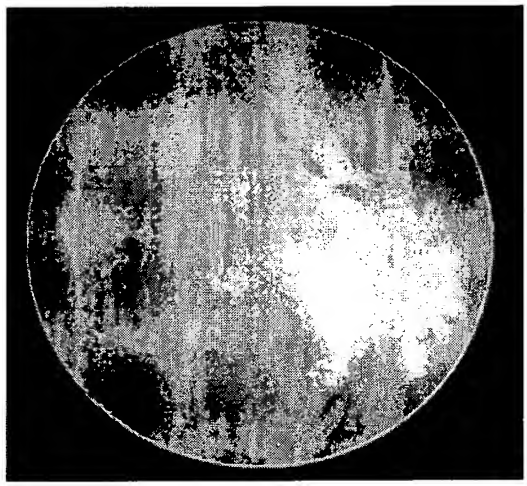

Abb. 2 ▶ **Untypische Manifestation einer okulären Toxoplasmose bei einer 58-jährigen Patientin. Die Diagnose konnte durch den Nachweis lokaler Antikörperproduktion gegen Toxoplasma gondii eindeutig gesichert und erfolgreich therapiert werden**

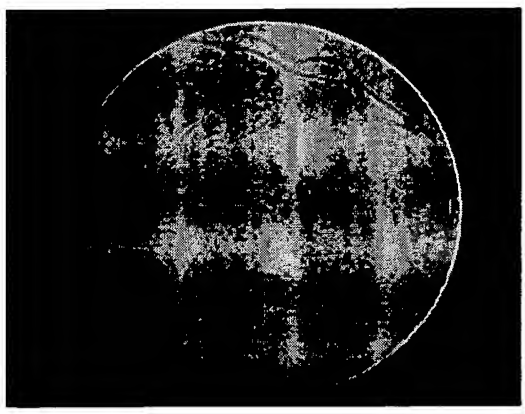

Abb. 3 ▶ **Fundusbefund bei einem 14-jährigen Patienten mit nicht charakteristischem klinischen Bild. Eine okuläre Toxoplasmose wurde durch die Kammerwasseranalyse diagnostiziert und erfolgreich mit einer antibiotischen Therapie behandelt**

scheinlich die PCR-Methode überlegen ist, scheint im weiteren Verlauf der Entzündung die Bestimmung der Antikörper vorteilhafter zu sein [1].

Die PCR beinhaltet einen höheren technischen Aufwand und erscheint störanfälliger. Insbesondere die kontaminationsfreie Entnahme der Probe ist wichtig, da häufiger falsch-positive Ergebnisse resultieren können. Mehrere Untersuchungen [2, 6] zeigten, dass die PCR-Untersuchung der Kammerwasserflüssigkeit bei der Toxoplasmose-Infektion des Auges zwar eine hohe Spezifität, aber eine nur geringe Sensitivität, besitzt [6]. Eine deutlich höhere Sensitivität wurde bei der PCR-Untersuchung von Glaskörper auf Toxoplasma gondii beobachtet [15]. Die Indikation zur Glaskörperpunktion wird jedoch eher kritischer gestellt und als komplikationsreicher gegenüber der Vorderkammerpunktion beurteilt. Insgesamt scheinen sich die Antikörperuntersuchung und die PCR-Analyse zu ergänzen, abhängig von der zugrunde liegenden Erkrankung und dem Immunstatus der Patienten [5].

Der serologische Nachweis einer Toxoplasmose-Exposition ist ein weit verbreitetes diagnostisches Verfahren. Aufgrund der hohen Durchseuchungsrate der Bevölkerung mit Toxoplasma gondii und der damit verbundenen häufig positiven Befunde der Serologie, wurde bislang bei okulärer Toxoplasmose oft nur ein negatives Resultat zum Ausschluss einer Toxoplasmose verwendet. Ein positiver Antikörpertiter wurde aber nur selten zur Erhärtung der Diagnose einer Toxoplasmose eingesetzt [19].

Unsere Untersuchung bestätigt diese Erfahrungen. Lediglich 4 Patienten in der Kontrollgruppe waren für IgG negativ. Zwar wird im akuten Entzündungsverlauf dem Nachweis von IgM als Immunglobulin der Frühantwort stärkere Bedeutung beigemessen, jedoch war nur bei 3 von 50 unserer Patienten ein positiver IgM-Titer zu beobachten. Der serologische Nachweis von Toxoplasmose-Antikörper ist daher als weniger aussagekräftig zu werten. Jedoch ist zu beachten, dass bei der Bewertung eines solchen Befundes die unterschiedliche Durchseuchung in der Normalbevölkerung zugrunde gelegt wird [3].

Auch ist zu berücksichtigen, dass wir den Serum-Titer nicht im Verlauf untersucht haben, da eine Serokonversion oder ein signifikanter Anstieg des Antikörpertiters zum Teil erst nach Wochen eintritt und sich damit die Diagnosestellung verzögert. Andererseits könnte bei länger zurückliegender Infektion ein signifikanter Titeranstieg ausbleiben und zu einem falsch-negativen Resultat führen. Unberücksichtigt blieb in unserer Untersuchung die Titerhöhe, der zur Beurteilung des Aktivitätsgrads eine Bedeutung beigemessen wird [3].

Fazit für die Praxis

Zusammenfassend ist festzuhalten, dass die Kammerwasseruntersuchung bei der okulären Toxoplasmose einen höheren Aussagewert gegenüber der Serumuntersuchung besitzt [11, 18]. Darüber hinaus haben Untersuchungen gezeigt, dass sich das spezifische Antikörpermuster bei okulärer Toxoplasmose im Vergleich zwischen Kammerwasser und Serum, insbesondere bei chronisch rezidivierendem Verlauf, unterscheiden kann [12]. Immunoblot-Untersuchungen konnten belegen, dass sich vermutlich aufgrund der lokalen Persistenz von Tachyzoiten bzw. Bradyzoiten ein differierendes Antikörperrepertoir in Kammerwasser und Serum ergibt.
Es bleibt stets zu berücksichtigen, dass es sich bei der Kammerwasserpunktion um ein invasives diagnostisches Verfahren handelt, bei dem Nutzen und Risiken gegeneinander abgewogen werden müssen. Wir beobachteten bisher bei keinem unserer Patienten durch die Punktion resultierende Komplikationen. Dennoch sollte der Eingriff ausgewählten Patienten mit unklarer Diagnose und therapeutischer Konsequenz vorbehalten bleiben.

Danksagung. Wir danken Frau Dr. Bollmann (Institut für Mikrobiologie) für ihre Unterstützung bei der serologischen Analyse der Toxoplasmose-Antikörperbestimmung.

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Aqueous humor analysis as a diagnostic tool in toxoplasma uveitis

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Key words: toxoplasma, uveitis, aqueous humor, diagnostic test, AIDS

Abstract

Analysis of local toxoplasma antibody production to confirm a suspected clinical diagnosis of toxoplasma chorioretinitis is a valuable diagnostic tool. Determination of toxoplasma antibodies in the blood of the patient is of limited use. When blood toxoplasma tests are negative this indicates that toxoplasma as a causative organism in the pathogenesis of uveitis is unlikely.

A positive blood test is a sensitive test (100% patients positive) but not a specific test since so many healthy individuals already have undergone subclinical infection and have acquired humoral immunity against the parasite.

We analysed 93 paired aqueous and serum samples for toxoplasma antibodies and total IgG and determined the Goldmann-Wittmer coefficient. In patients retrospectively diagnosed as having toxoplasma chorioretinitis 16 out of 22 had a positive coefficient, indicating local parasite antibody production. In one patient with AIDS we also found a positive toxoplasma coefficient. Three out of 15 patients with posterior uveitis of unknown origin also had a positive coefficient. None of the cataract patients tested (n = 32) had a positive coefficient. Major drawbacks of aqueous humor analysis are that a false negative antibody coefficient can occur when a massive blood aqueous barrier breakdown has occurred.

Introduction

Ocular toxoplasmosis is currently the most frequent cause of posterior uveitis and is considered a late manifestation of a congenital infection [1, 2, 3].

The fact that the parasite resides in the retina during development of the embryo and can remain there for years without causing problems to the visual system is an important fact which should be considered when interpreting serological examinations. The peak age of patients presenting in the clinic with visual problems is in the third decade (Fig. 1). The diagnosis of ocular toxoplasmosis is based on the characteristic clinical picture of a focal necrotizing retinitis.

Detection of toxoplasma antibodies in the blood of these cases is a confirmation of the clinical diagnosis. In a number of patients the clinical picture may be atypical or funduscopy may not be possible due to the inflammatory response in the vitreous. Various other causes should then be considered in the differential diagnosis. The presence of circulating toxoplasmal antibodies is however not of great importance in view of the fact that many healthy individuals also have these antibodies resulting in a low specific diagnostic value. The absence of circu-

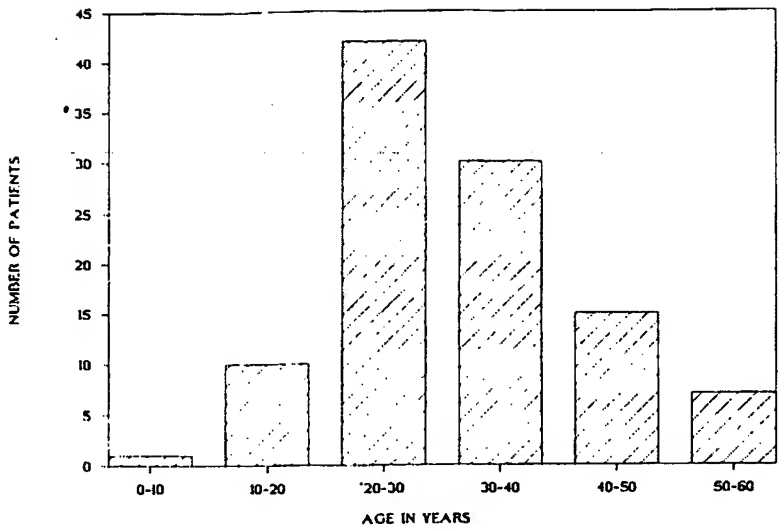

Fig. 1. Age of patients with toxoplasma chorioretinitis.

lating antibodies on the other hand makes the diagnosis of ocular toxoplasmosis improbable [4, 5].

In patients, having detectable serum toxoplasma antibodies, a suspected toxoplasma chorioretinitis can be confirmed by analysis of intraocular parasite antibody production. In the following sections details of the technique and interpretation of results will be discussed.

Indication

In patients with the typical clinical picture of a focal necrotizing lesion characteristic for toxoplasma chorioretinitis, additional laboratory testing is not needed. The lesions heal with time and large atrophic scars remain.

Not all cases present as outlined above. Active retinitis may lead to hemorrhage and sheathing of blood vessels. Furthermore the overlying vitreous may be involved. Also, the typical toxoplasma satellite lesions adjacent to old scars are not always seen. To establish the diagnosis of a suspected toxoplasmosis, aqueous humor testing is a valuable diagnostic procedure. This may be preceded by first testing the serum for toxoplasma antibodies. If negative it is unlikely that the patient will have ocular toxoplasmosis [4, 5]. If positive one may proceed and also collect aqueous humor. A sample of venous blood should be taken simultaneously.

Toxoplasma antibodies

Analysis of toxoplasma antibodies can be determined using various techniques. We have chosen for an immunofluorescence technique whereby only small amounts of aqueous humor are needed.

Formalin fixed toxoplasma organisms (Behring, Hoechst) are coated onto glass slides (Nutacon) and air dried. Subsequently 10 μl of aqueous humor (undiluted, 1/2, 1/4 etc.) and the same volume of various dilutions of the paired serum sample are incubated on one slide. A positive reference and negative control are included on the slide.

After incubation for 30 minutes at 37°C the slides are washed and incubated with a fluorescein labeled antibody against human IgG (Behring, Hoechst).

After incubation (30 minutes) and washing the result is scored with a Leitz Ploemopak fluorescence microscope. The titre of antibodies is defined as the highest dilution giving a positive result in the test. Total IgG levels in serum and aqueous are measured using radial immunodiffusion analysis.

Local synthesis toxoplasma antibodies

Antibodies detected in the aqueous can originate from the peripheral blood or from within the eye. Intraocular synthesis of antibodies is considered to have taken place if the relative amount of toxoplasma antibodies compared to the total immunoglobulin level found in the aqueous exceeds that measured in a paired serum sample. The quotient of the relative amount of toxoplasma antibodies in the aqueous and serum is named the Goldmann-Witmer coefficient:

$$\frac{\text{antibody titre aqueous}}{\text{total immunoglobulin aqueous}} : \frac{\text{antibody titre serum}}{\text{total immunoglobulin serum}}$$

Theoretically a coefficient above 1.0 would indicate a local antibody production within the eye. In view of the variability in the results of the various measurements, an antibody coefficient above 3.0 is considered significant. An example of a representative case is shown in Table 1. This patient presented with a retinitis in the macular region at the age of 14. No diagnosis was made at that time and the retinitis subsided. Four years later the same lesion reactivated. An aqueous tap was performed and a blood sample was taken. No toxocara antibodies were detectable in serum. Analysis of toxoplasma and herpes simplex virus antibodies showed detectable titres in aqueous for both organisms. The antibodies against toxoplasma were due to local intraocular production (coefficient 23) whereas the antibodies against the herpes simplex virus were due to blood aqueous barrier breakdown (coefficient 0.9). This example shows that an isolated aqueous humor sample testing does not provide adequate information unless a paired serum sample is available. When using this test in cases where the clinical diagnosis of ocular toxoplasmosis was not clear, we found a positive test in 70% of patients retrospectively considered to have a toxoplasma chorioretinitis (Table 2). Of the 16 patients showing intraocular antibody production, 10 had a coefficient between 3 and 10, 4 had a coefficient between 10 and 50 and 2 had coefficients above 50.

A positive coefficient was also calculated in three cases of posterior uveitis, in whom further clinical data were not yet available at the time of writing. In one case of retinitis in an AIDS patient a coefficient of 5 was calculated for toxoplasma. No antibodies against CMV could be detected in the aqueous of this patient. All aqueous samples obtained during cataract extraction were negative.

In a number of non-toxoplasma uveitis cases, detectable titres could be measured in aqueous. Determination of total immunoglobulins and comparison with the serum titre however indicated that the aqueous humor toxoplasma antibodies were derived from the blood, resulting in an antibody coefficient lower than 3.

In cases where there is a massive breakdown of the blood aqueous barrier and where the titre of antibodies in the serum is high, a possible intraocular production of antibodies may be masked, lead-

Table 1. Aqueous humor analysis as a diagnostic aid in toxoplasma chorioretinitis.

Patient S. Presenting with retinitis Toxocara? Toxoplasma? Viruses?	Titer		Coefficient
	Aqueous	Serum	IgG
toxoplasma	1/4	1/32	23
herpes simplex	1/2	1/400	0.9
total IgG mg/ml	0.043	7.9	

Conclusion: Intraocular production toxoplasma antibodies.

Table 2. Intraocular production of toxoplasma antibodies in the eye.

	toxoplasma antibody negative < 3	coefficient positive > 3
toxoplasma chorioretinitis	6	16
posterior uveitis e.c.i.	12	3
retinitis (AIDS)	6	1
pan uveitis	5	0
Fuchs cyclitis	8	0
cataract	32	0
miscellaneous	4	0

Data represent number of patients.

ing to a false negative Goldmann-Witmer coefficient.

Furthermore it is possible that the degree of local antibody production is dependent on the activity of the chorioretinitis as well as upon the number of recurrences. A false negative coefficient may thus be obtained when an aqueous sample is taken from a quiet eye or during the first episode of toxoplasma uveitis. Future analysis of data will prove whether the above mentioned hypothesis is correct.

A false positive coefficient may also theoretically be possible. Polyclonal stimulation of lymphocytes in the uvea may lead to a shift in the balance of various antibody populations in the eye compared to the blood compartment. To rule out polyclonal activation one may analyze a coefficient for two different antigens and divide these two.

Future developments

Determination of local toxoplasma antibody production is indirect proof of the presence of the parasite within the eye. Direct proof would involve the detection of the parasite itself or its' antigens in an aqueous sample. Although sensitive immunoassays are available to test the free toxoplasma antigen these methods have not yet been applied to aqueous humor. In future it might also be possible to detect the presence of toxoplasma DNA using PCR techniques.

New developments in the analysis of local production of antibodies include analysis of oligoclonal bands by iso-electric focussing and more efficient immunoassay techniques. The use of so-called 'antibody capture' techniques may provide a rapid test result in the near future.

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CLINICAL INVESTIGATION

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Intraocular antibody production in intraocular inflammation

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Abstract *Background:* The production of intraocular antibodies is considered a specific marker for active infectious uveitis. The aim of our study was to evaluate the diagnostic value of aqueous humor analysis in consecutive patients referred to a tertiary clinical center. *Methods:* We analyzed 91 paired aqueous humor/serum samples from 89 patients with intraocular inflammation. In 71 patients aqueous humor analysis was used as a positive or negative confirmation of the suspected cause, whereas in 18 patients the clinical diagnosis was completely uncertain. A modified micro-ELISA technique was used to detect intraocular IgG production against Toxoplasma gondii, varicella zoster virus, herpes simplex virus and cytomegalovirus. Statistical analysis was performed using the "Cohen's kappa" test. *Results:* Specific intra-ocular antibody production could be detected in 12 (66.7%) of 18 patients with uncertain diagnosis. Subsequently initiated therapy led to clinical improvement in 10 patients, whereas 2 patients remained unchanged. In 2 (2.8%) of 71 patients aqueous humor analysis led to revision of the initially suspected etiology and to a change of therapy. Statistical analysis showed a significant accordance of diagnosis and aqueous humor analysis ($P<0.01$). *Conclusion:* In patients with infectious uveitis, analysis of intraocular synthesis of specific antibodies is a valuable tool to establish the etiology rapidly and allows initiation of targeted antimicrobial treatment.

Introduction

Infectious uveitis remains a significant cause of visual impairment that can often be prevented by early instillation of adequate antimicrobial therapy. Commonly the diagnosis is based on clinical findings; however, additional laboratory data for confirmation are desirable.

As early as in 1902 van Dungern [9] was successful in detecting intraocular production of antibodies. More than 40 years ago Goldmann and Witmer recommended analysis of the aqueous humor in cases of uncertain uveitis [13]. Based on a protein gradient between serum and aqueous humor they introduced the "Witmer coefficient" indicating local immunoglobulin production.

Since then modifications have been established for detection of locally produced antibodies in the aqueous humor of patients with intraocular inflammation [5, 10, 25]. For more than 10 years the detection of specific DNA in the aqueous humor by polymerase chain reaction (PCR) has been increasingly applied in uveitis [3, 7, 12, 29]. However, this method is more expensive than measuring antibodies in aqueous humor or serum. Moreover, antibody determination is comparable to PCR regarding specificity and sensitivity [7, 8, 24] but is less expensive.

However, analysis of aqueous humor is still not routinely used in diagnosis of suspected infectious intraocular inflammation, although definitive diagnosis might be difficult.

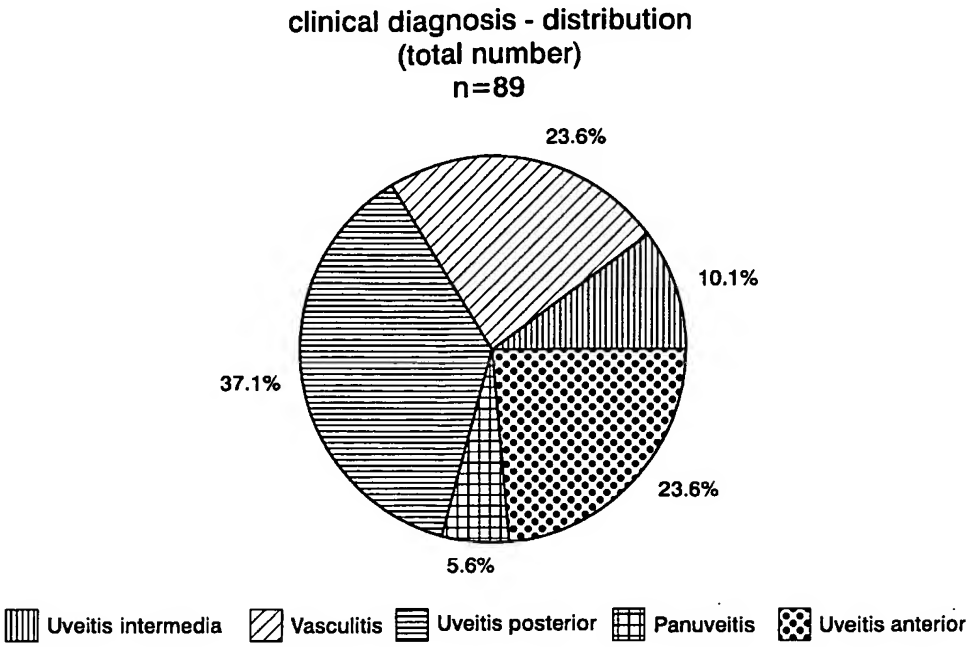

Fig. 1 Clinical diagnosis of all patients (n=89) according to the criteria of the International Uveitis Study Group [6]

In 1983 Hartmann et al. [15] introduced a modified micro-ELISA technique, based on cerebrospinal fluid diagnosis [11] and adapted to aqueous humor conditions, for determining intraocular antibody production. Since we use this method as a diagnostic means in selected patients with uveitis we sought to evaluate its diagnostic value.

Methods

Patients' characteristics

Between May 1996 and January 1998, 91 samples of aqueous humor were obtained from 89 patients with intraocular inflammation. Patients were recruited from our outpatient clinic for inflammatory eye diseases. The diagnosis was based on clinical characteristics according to the criteria of the International Uveitis Study Group [6]. Diagnoses were: anterior uveitis (21), intermediate uveitis (9), posterior uveitis (33), vasculitis (21) and panuveitis (5) (Fig. 1). In 18 patients (20.2%) the clinical diagnosis could not be established on clinical grounds or from ancillary tests. Distribution of diagnoses is outlined in Fig. 2. In the remaining 71 patients (79.8%) aqueous humor analysis was used as a positive or negative confirmation of the suspected causes. Specific etiologic diagnoses in these patients were: toxoplasmosis retinochorioiditis (32), acute retinal necrosis (5), cytomegalovirus retinitis (5), herpetic uveitis/iridocyclitis (24), Fuchs' heterochromic uveitis (4) and Vogt-Koyanagi-Harada syndrome (1).

Analysis of intraocular antibody synthesis

Blood and aqueous humor samples were collected simultaneously. Approximately 0.1–0.3 ml of aqueous humor was aspirated. Aqueous humor was obtained in topical anesthesia by performing a paracentesis under microscopic control using a 30-G syringe.

We used a modified micro-ELISA technique to detect intraocular IgG production as previously mentioned [15]. Antibodies against Toxoplasma gondii, varicella zoster virus, herpes simplex virus and cytomegalovirus were measured (Dade Behring Marburg, Marburg, Germany) in aqueous humor samples and serum samples to confirm local antibody production, assuming these agents to be the most common causes for infectious uveitis. The aqueous humor and serum samples were diluted to an equal total IgG concentration. A minimum of 1.25 µl–130 µl (average 35.25 µl) of aqueous humor, depending on individual total IgG concentration in aqueous humor, was needed to measure the antibodies mentioned. Local antibody production in aqueous humor was defined as a positive antibody reaction in a higher grade of dilution than in the serum sample of the same patient. It is expressed as the lowest IgG concentration (mg/dl) at which specific IgG is still detectable. Using this method there is no need for formulas, factors or coefficients (Fig. 3).

Statistical analysis

For statistical analysis we considered the interrater agreement on two ratings (clinical diagnosis and aqueous humor analysis) and categorical scales. The "Kappa statistics" [14], a composite measure of agreement across all categories, was used.

Results

No postoperative complication was seen in any patient following anterior chamber tap. In 12 (66.7%) of 18 uncertain cases aqueous humor analysis led to a specific diagnosis. The specific antibody production patterns found in these patients are shown in Table 1. Specific therapy was initiated and 10 patients improved during follow-up, while 2 patients remained unchanged. In the remaining 6 of these 18 patients the analysis provided no clue to the diagnosis.

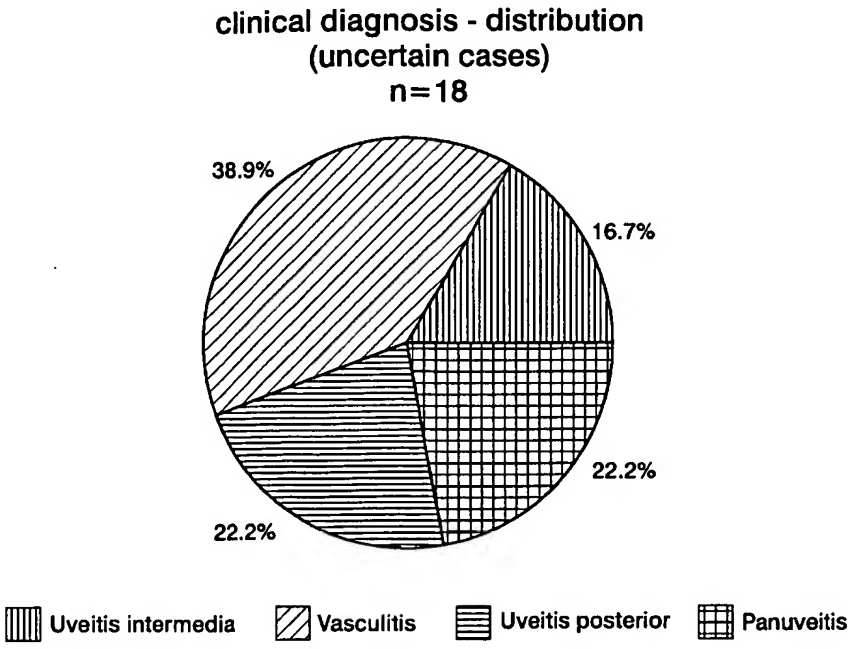

Fig. 2 Distribution of uncertain clinical diagnosis in 18 patients

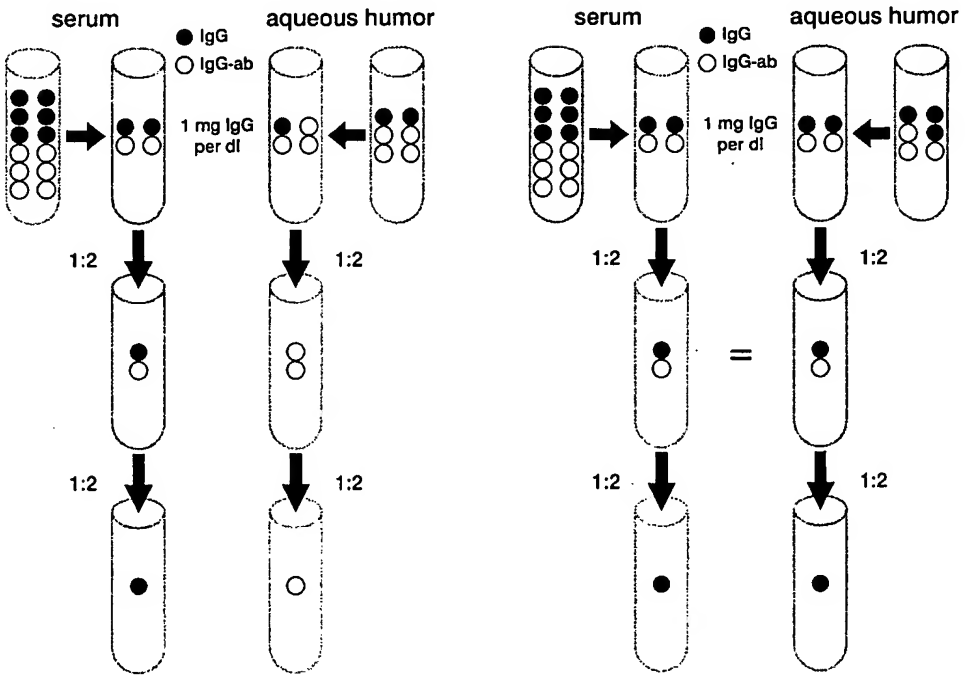

Fig. 3 Modified micro ELISA technique for determing local antibody production in aqueous humor. Left: A pathologic result with local antibody production. Right: A normal result. IgG nonspecific IgG; IgG-ab specific IgG

In 2 (2.8%) of 71 patients with assumed certain clinical diagnosis, aqueous humor analysis led to a revision of the diagnosis and to a change of therapy that brought about subsequent clinical improvement. The first patient was diagnosed as affected by acute posterior multifocal placoid pigment epitheliopathy. Her visual acuity was 0.3 at first visit. She was treated with systemic steroids without improvement of the disease. Aqueous humor analysis revealed antibodies against varicella zoster virus. Specific treatment with acyclovir led to improvement and stabilization of the condition; visual acuity improved to 0.6. The second patient was diagnosed as having herpetic keratouveitis. Aqueous humor analysis was negative for specific antibody production. Further diagnostic tests were applied and culture established the diagnosis of acanthamoeba infection. A subsequent adjust-

Table 1 Specific diagnosis was established in 12/18 uncertain cases

Local antibody production	n
Toxoplasma gondii	3
Varizella zoster virus	3
Herpes simplex virus	4
Cytomegalovirus	0
Herpes simplex virus and varicella zoster virus	2

Table 2 False-positive results in clinically certain diagnoses (3/71)

Diagnosis	Aqueous humor analysis
Fuchs' heterochromic uveitis	Toxoplasmosis gondii
Toxoplasmosis retinochorioiditis	Herpes simplex virus
Vogt-Koyanagi-Harada syndrome	Cytomegalovirus

Table 3 False-negative results in clinically certain diagnoses (3/71)

Diagnosis	Aqueous humor analysis
Herpetic iridocyclitis	Negative
Toxoplasmosis retinochorioiditis	Herpes simplex virus
Cytomegalovirus retinitis	Negative

ment of therapy from acyclovir to chlorhexidine resulted in clinical improvement and an increase in visual acuity from 0.2 to 0.5.

In 3 of these 71 patients (4.2%), specific antibody synthesis was detected but was not compatible with the clinical manifestation (false positive). The specific diagnoses and aqueous humor analyses of these patients are shown in Table 2. In 3 of those 71 patients (4.2%) aqueous humor analysis did not confirm the clinical diagnosis, although it was verified by positive treatment response (false negative). The specific diagnoses and aqueous humor analyses of these patients are shown in Table 3.

In patients with specific local antibody production that matched the presumed clinical diagnosis the following relation between antibody findings and classified subtypes of uveitis could be observed: Antibodies against Toxoplasma gondii (n=33) and cytomegalovirus (n=1) were all detected in patients with posterior uveitis. A specific immune response against varicella zoster virus was predominantly detected in patients with vasculitis (57.1%), but also in patients with anterior uveitis (28.6%) and posterior uveitis (14.3%). A specific immune response against herpes simplex virus was mostly detected in patients with anterior uveitis (64.3%), followed by patients with vasculitis (28.6%) and posterior uveitis (14.3%).

On statistical analysis the "observed Cohen's kappa" value is 75.82% and the chance corrected Cohen's kappa is 49.03% with a standard error of 0.09340 and a confidence interval of [0.3073; 0.6734]. This indicates a statistically significant accordance of diagnosis and aqueous humor analysis by our method ($P<0.01$).

Discussion

This study revealed that specific intraocular IgG antibody analysis is an important diagnostic tool for detection, confirmation or exclusion of infectious uveitis.

In this investigation aqueous humor analysis for determing local IgG production in selected uveitis patients reveals high accordance between analysis results and the clinical course of specific intraocular inflammation. We preferred a modified ELISA technique to detect specific antibody production on grounds of its high sensitivity and specificity, and because there is no need to correct for a reference protein or to determine a coefficient. This method seems to be very helpful in the diagnosis of uncertain cases of uveitis, with a 66.7% success rate. It is also helpful in confirming the clinical diagnosis, while 2.8% of these cases had to be revised by aqueous humor analysis. Our method shows high accordance between aqueous humor analysis and diagnosis. The high specificity and sensitivity of our method were shown in earlier studies by constituting a control group with cataract patients [15, 16].

Our results are at least as reliable as those of other studies. To date aqueous humor analyses of local antibody production in uveitis have had no more than 55% accordance with clinical diagnosis [7, 8, 19, 24]. Obviously, critical inclusion criteria play an important role for the success of aqueous humor analysis. Our patients were selected only by experienced specialists in uveitis at our clinic. In previous studies patients were not selected for special indication to infectious uveitis [8], or ocular fluid samples were referred from external clinics [19]. That might explain the difference in the results.

Comparing our method of antibody measurement to the PCR method, both seem to have certain advantages. The sampling interval between infection, immune response and aqueous humor tap is important [23]. Whereas in the early state of infection PCR is probably superior, the measurement of intraocular antibodies becomes more advantageous during the course of inflammation [1]. In addition, the high sensitivity of the PCR method increases the risk of contamination and false-positive results. For additional certainty analysis of aqueous humor might be performed using both techniques.

It is also important to differentiate between immunocompetent and immunocompromised patients, e.g. patients with acquired immunodeficiency syndrome (AIDS). Only one patient in our study had AIDS. The

clinical certain diagnosis was cytomegalovirus retinitis, but aqueous humor analysis did not reveal local antibody production against cytomegalovirus, although specific treatment led to clinical improvement. Consistently negative results in aqueous humor samples of AIDS patients or patients with cytomegalovirus retinitis for specific antibody production have been described by other authors [1, 7]. For those patients detection of the viral antigen seems to be superior. However, in immunocompetent patients PCR not only takes more time and is more costly but also seems to be inferior to methods of analyzing local antibody production in aqueous humor [7, 8]. In particular, the sensitivity of PCR is reduced by the low volume of the samples [3]. Using our method even a volume of 100–200 µl aqueous humor allows determination of a panel of specific antibodies.

Nevertheless we had some false-negative and false-positive results. The proportions corresponds with data from other authors [24]. Aqueous humor analysis is not expected to be efficacious in patients with severe blood-aqueous humor barrier dysfunction. That might be a reason for false-negative results. False-positive results are probably caused by polyclonal activation of B lymphocytes that may, on balance, shift the antibody production towards the eye and away from the blood compartment [21, 28].

We found a false-positive result in a patient with Fuchs' heterochromic iridocyclitis. Aqueous humor analysis revealed local antibody production against Toxoplasma gondii. Although an association between Fuchs' heterochromic iridocyclitis and toxoplasmosis has been described, and Toxoplasma gondii has even been discussed as a possible etiologic agent for this iridocyclitis [2, 26], we interpreted our result as false-positive as Hey et al. [18] denied a proved association between those two entities.

Aqueous humor analysis allows multiple tests, but still it is limited by the small volume of material one can collect from the anterior chamber. Therefore it is necessary to analyze the common known etiologic agents for infectious uveitis. Some authors also include testing for Epstein-Barr virus [8], but Ongkosuwito et al. [22] found no indication of an important role of this virus in the pathogenesis of intraocular inflammation.

Any invasive procedure has to be judged by its risks and benefits. A carefully performed paracentesis and collecting of aqueous humor involves little additional danger to the patient. There is one systematic retrospective study addressing the potential risks of anterior chamber paracentesis [20]. In that series of 361 patients no serious side effects such as cataract, keratitis or endophthalmitis were observed. Although we also did not see any complications one has to consider a possible risk of infection, especially in patients with good visual acuity [4, 17].

Early and correct treatment of infectious uveitis is important for the prognosis and may have long-term consequences. Lately it has been shown that significant reduction of recurrent herpetic keratouveitis can be achieved by a 12-month treatment with acyclovir [27]. This is decisive for the patient and makes exact diagnosis even more important. Therefore, ophthalmologists should consider performing aqueous humor analysis in selected cases of uveitis.

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Analysis of the Antibody Repertoire in Tears of Dry-Eye Patients

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Key Words
Dry-eye disease · Tears · IgA antibody repertoire

Abstract
Purpose: It has recently been suggested that dry-eye disease has an underlying autoimmune mechanism. This hypothesis is further supported by the successful treatment of the disease with immunomodulatory drugs such as cyclosporin A. Although it is known that tears contain antibodies, very little is known about the antibody repertoires in tears. It was the aim of this study to analyze the IgA antibody repertoire against ocular antigens in the tears of patients suffering from dry-eye disease and compare it to those of healthy volunteers. ***Methods:*** Two groups were examined: 20 healthy volunteers (controls) and 28 patients suffering from dry-eye disease. The patients were grouped according to the results of the basic secretory test. Patients with values ≤ 10 and subjective symptoms were classified as dry-eye patients. All tears were tested against Western blots of ocular antigens. For each Western blot, a densitograph was built by digital image analysis, and subsequently a multivariate discriminate analysis was performed. ***Results:*** A complex staining pattern was found in the tears of both dry-eye patients and healthy controls. However, the number of peaks was statistically significantly increased ($p < 0.05$) in the tears of dry-eye patients. The discriminant analysis found a statistically significant difference between the antibody repertoires of both groups (Wilks' $\lambda = 0.11$; $p < 0.001$). ***Conclusions:*** In this study, it could be shown that the complex antibody repertoires in the tears of patients suffering from dry-eye disease are different from those found in the tears of healthy volunteers. Thus, our findings support the hypothesis that the dry eye disease has an autoimmune mechanism.

Introduction

The dry-eye syndrome is one of the most frequent eye diseases in the industrial world. In the USA, 1 of 5 Americans, i.e. 59 million patients, suffers from this disease (Eagle Vision, Yankelovich and Partners, 1997). The number of dry-eye patients has been doubled during the last 10 years [1–4]. The knowledge of the pathogenesis of tear deficiencies has grown significantly. However, the diagnosis and treatment of the dry-eye syndrome are still challenging and until now, a causative therapy of this disease does not exist. 'Dry eye' describes a disease with various symptoms resulting from aqueous, mucin or lipid defi-

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ciency. On the one hand, this deficiency can be caused by different diseases such as Sjörgen's syndrome; on the other hand, most patients do not have any accompanying disorders [5, 6].

Dry-eye patients typically suffer from discomfort, burning, irritation, photophobia, blurred vision and have an increased risk of corneal infection and thus irreversible tissue damage [7, 8]. The clinical diagnosis of 'dry eyes' is commonly based on the patient's subjective symptoms, the patient's history, slitlamp examination, determination of tear film breakup time and the so-called basic secretory test (BST); however, there is only a poor correlation [9, 10], which is not only poor between the different tests, but also between the test results and the course of the disease. More recently, the electrophoretic analysis of tear proteins gained attention for the diagnosis of dry eyes. A new analytical procedure was developed for the analysis of tear protein patterns as a diagnostic tool for the detection of dry eyes. This procedure is based on digital image analysis of the electrophoretic patterns of tear proteins and subsequent calculations using multivariate statistics [11–15].

The treatment of dry-eye disease is currently limited to the 'palliative' use of artificial tears. Recently, it has been discussed whether dry-eye disease is caused by a cytokine- and receptor-mediated inflammation [16–18]. It was hypothesized that this inflammatory process can lead to the expression of major histocompatibility complex class II molecules that could trigger an autoimmune response [16, 19]. Because of this possible immune-based inflammatory pathomechanism of dry-eye disease, a treatment of the disorder with immunomodulatory agents was suggested. The topical treatment with cyclosporin A resulted in significant improvements in the signs and symptoms of the disease [20–22]. Thus, these studies give more evidence for the autoimmune nature of this disease. Furthermore, it has recently been shown that the human conjunctiva and lacrimal drainage system have an associated lymphoid tissue that is capable of detecting antigens and of inducing a complete immune response by the activation of lymphatic cells and the secretion of antibodies [23].

Although it has been shown that antibodies, especially IgA antibodies, are present in tears [24–26], only little is known about the composition of these antibodies. Because of the possible autoimmune nature of dry-eye disease, it can be suggested that a subset of these autoantibodies exerts reactions against ocular antigens. In the present study we used a wide range of ocular antigens deriving from the protein separation of crude retinal extract. The antigen-antibody reactions were analyzed by Western blotting followed by a digital image analysis. The search for pathological autoantibodies is complicated by the fact that even normal sera contain large repertoires of naturally occurring autoantibodies against many different autoantigens [27, 28]. In the present study, multivariate statistical techniques based on the densitographs of each Western blot were used to detect differences in the distribution of antibodies against ocular antigens in tears. Recently, this technique has been successfully used in myasthenia gravis, Graves' disease and experimental uveitis [29–31].

The aim of this study was to analyze the IgA autoantibody repertoires against ocular antigens in the tear fluid of dry-eye patients and to compare them to those of healthy subjects.

Patients and Methods

Tears were obtained from 48 subjects: patients with dry-eye symptoms (n = 28) and healthy volunteers (n = 20).

The tears were sampled using a 5-µl glass capillary. Thereafter, the tears (volume: approx. 5 µl) were stored at –70°C until use. The BST was performed. The initial clinical diagnosis of 'dry eye' was based on the BST value: patients were categorized as 'dry-eye group' with a BST value ≤10 mm and more than two of the subjective symptoms such as discomfort, foreign-body sensation, burning, irritation, photophobia or blurred vision. There were no patients suffering from Sjögren's syndrome or other systemic diseases leading to dry eyes included in this study.

Biochemical Procedures

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis. Crude bovine retinal extract (1.5 mg/ml) was prepared as described before [32]. The retinal proteins were redissolved in sample buffer (0.08 *M* Tris-HCl, pH 6.8, 1% v/v 2-mercaptoethanol, 10% w/v saccharose, 1% SDS, 0.005% bromophenol blue) and separated by SDS-PAGE on discontinuous slab gels [33] (stacking gel: T = 6%, C = 2.5%, 0.05 *M* Tris-HCl, pH 6.8, 0.1% w/v SDS; separating gel: T = 12.8%, C = 2.5%, 0.0375 *M* Tris-HCl, pH 8.8, 0.1% SDS; electrode buffer: 0.188 *M* glycine, 0.188 *M* Tris, pH 8.8, 0.1% SDS; approx. 25 µg retinal protein/lane).

Western Blotting. The SDS-PAGE preparations were electroblotted onto a nitrocellulose membrane using the Semi-Dry Blotter (Biometra, Göttingen, Germany) [34]. The quality of transfer was checked by staining the membrane with avidin-biotin (BioRad, Munich, Germany). The membrane (Western blot, WB) was cut into strips approximately 0.4 cm in width. Tear samples were centrifuged at 12,000 *g* for 3–5 min. The strips were incubated for 12 h with 0.5 µl of tear samples [diluted 1:40 in 5% bovine serum albumin (BSA), in Tris-buffered saline (TBS)]. After incubation, the WBs were washed, blocked with 10% BSA in TBS (1 h), incubated with peroxidase-conjugated rabbit anti-human-IgA serum (diluted 1:2,000 in 5% BSA in TBS, 1 h) and washed. The reaction product was visualized using 0.05% 4-chloro-1-naphthol with 0.015% hydro-

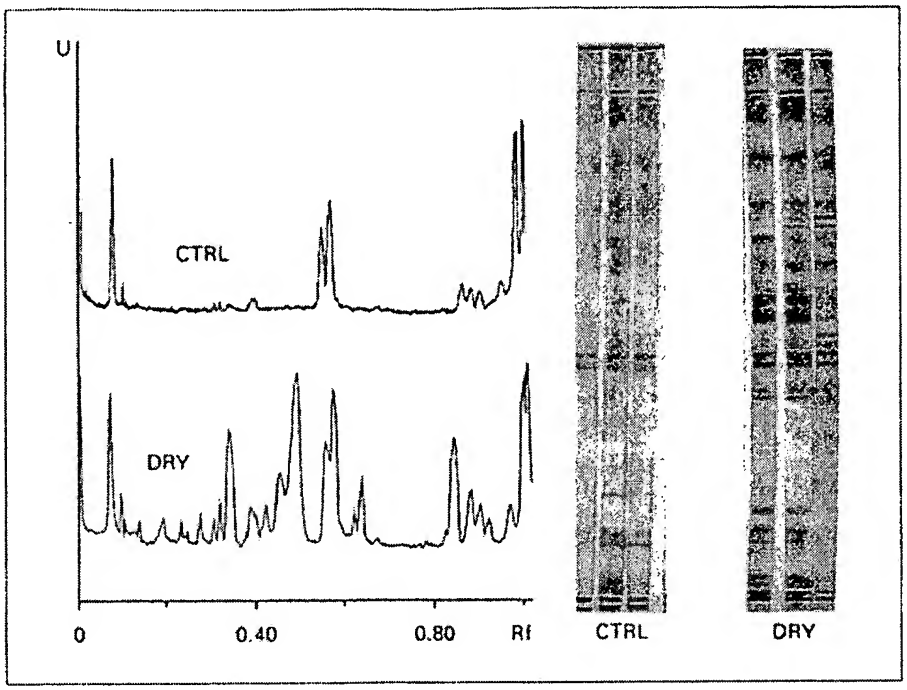

Fig. 1. Left: densitographs of the control (CTRL) and dry-eye (DRY) groups. Scanner units are plotted versus the Rf values. Right: photographs of WBs of the dry-eye and the control groups.

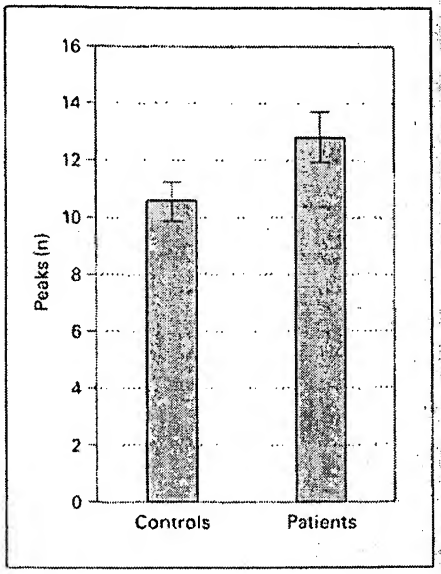

Fig. 2. Mean number of peaks (± standard error) in the tear fluid for both groups: patients suffering from dry-eye disease and healthy volunteers (controls). The number of peaks is significantly ($p < 0.05$) higher in the tears of patients compared to those of controls.

gen peroxide in 20% methanol in TBS. Molecular weights were estimated using marker proteins (Pharmacia, Freiburg, Germany; MW standard 'high' and 'low').

Digital Image Analysis of WBs

The data were acquired using a color flat-bed scanner (Epson GT-8000, Epson Germany, Düsseldorf, Germany). Digital image analysis and evaluation of densitometric data of the WBs were performed by means of ScanPacK® software (Biometra) as described in detail elsewhere [35–37]. For each WB strip, ScanPacK creates densitometric data files by showing the gray-intensity values versus the RF (relative mobility) values (x-axis). ScanPacK evaluates e.g. height, area, molecular weight and Rf value for all peaks in this densitometric data file.

Statistical Procedure

Each individual densitograph was reduced to 70 extinction values by first calculating the mean extinction values from the original 8-bit gray values of the densitograph file for each 1/70 of the region of interest (either across the entire Rf range, i.e. from Rf = 0 to Rf = 1, or within a relevant part of it). Thereafter the background level was subtracted from these data and the resulting maximum extinction (i.e. band intensity of an individual blot) was set to 100%. The other extinction values of this blot were transformed into relative percentage, thus reducing the influence of different absolute staining intensities of individual blots.

From these data vectors, a multivariate discriminant analysis was performed, which not only tests the null hypothesis that mean data vectors of the different groups derive from a multivariate normally distributed population, but also shows which of the various groups are statistically different. Based on this, discriminant function analysis can be used to determine which variables (Rf ranges) caused the mean value comparison to become significant or which variables can discriminate between groups. Additionally, the analysis allows a classification of blots; it can be used to test whether an individual blot pattern is similar to the pattern of a particularly known group or to which of several group patterns the greatest similarity exists. These procedures have been described in detail elsewhere [29, 30]. The statistical calculations were performed by Statistica® (Statsoft, Tulsa, Okla., USA).

Results

In the tear fluid of both controls and dry-eye patients, a complex staining pattern of antigen-antibody reactions against ocular antigens could be found. Figure 1 illustrates a densitograph and some photographs of WBs of both groups. It can be seen even visually that the complexity of the staining patterns is more expressed in the dry-eye group.

The mean number of peaks was statistically significantly ($p < 0.05$) increased in the WBs of tears of dry-eye patients compared to those of healthy controls (fig. 2).

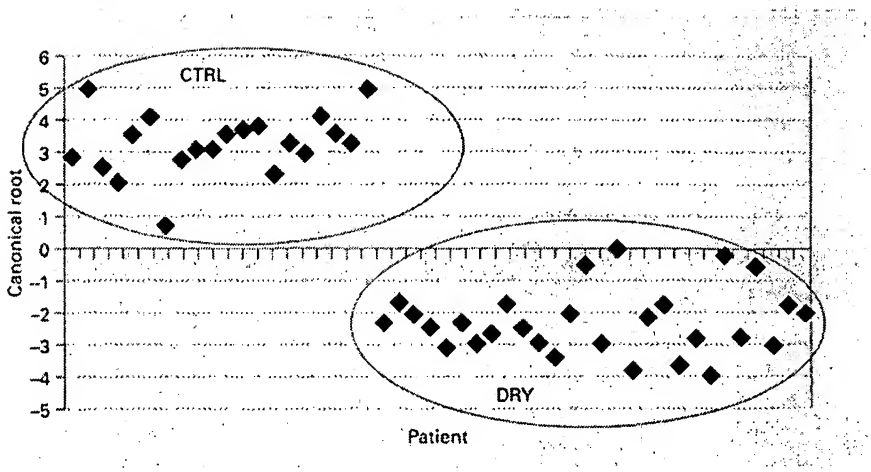

Fig. 3. Canonical roots derived from the discriminant analysis were plotted for each single patient. The figure illustrates the quality of discrimination: the closer the points to each other, the more similar the autoantibody patterns on the WBs. One can see a very sharp separation between both groups (DRY, CTRL) without any overlap.

To quantify any changes between the autoantibody patterns of both groups, a discriminant analysis was performed which revealed a highly significant difference between both groups (Wilk's $\lambda = 0.11$; $p < 0.0001$).

Figure 3 demonstrates the canonical roots for each WB of each patient. The canonical roots were calculated by the discriminant analysis. These canonical roots could give insight into the quality of separation between both groups: the closer the points in the figure, the more similar the autoantibody patterns. The figure reveals a very good and sharp separation between both groups without any overlap.

Discussion

In 1981, it has been demonstrated that tears contain antibodies, especially secretory IgA antibodies [6]. Recently, it could be shown that the lacrimal system has an associated lymphatic tissue [23]. Thus, it can be concluded that the antibodies in tears are not only the filtration product from the blood vessels, but are secreted by the immune tissue of the lacrimal system itself.

In other studies, an autoimmune process has been proposed as being responsible for the pathogenesis of dry-eye disease [20–22]. Thus, one can expect to find different autoantibody repertoires in the tears of healthy subjects and dry-eye patients. In this study we found complex IgA reactivities in the tear fluid of both normal volunteers and dry-eye patients.

Recently, we have demonstrated by means of electrophoretic techniques that the concentration of secretory IgA is decreased in the tears of dry-eye patients [15]. However, until now only little has been known about the reactivities of the IgA antibodies quantified in these electrophoretic studies.

The analysis of the IgA autoantibodies against ocular antigens is hampered by the facts that it is known that natural autoantibodies occur even in the serum of healthy persons and that the variety of autoantigens is very complex. Thus, in this study we used a method which can deal even with very complex antibody repertoires trying to find differences between them.

In the present paper, we found a more complex autoantibody repertoire revealing significantly more peaks (antigen-antibody reactions) in the tears of dry-eye patients as compared to controls. Additionally, the multivariate discriminant analysis could demonstrate a statistically significant difference between both autoantibody repertoires. This is in accordance with the knowledge about other autoimmune diseases. Using the method presented in this paper, the autoantibody repertoires in diseases such as myasthenia gravis, Graves' disease and the Tourette syndrome were different from those of healthy subjects [29, 30, 38]. It is common to all of these analyses that a large variety of the autoantibody patterns could be found in all patients making it (nearly) impossible to successfully distinguish just one or two antibodies being responsible or involved in the pathogenesis of the disease from the 'immunological noise'.

The present paper reveals very similar results: the autoantibody repertoire found in the tears of dry-eye patients were very different from those of controls, but actually it was impossible to determine a single molecular-weight region of immunological reactivity present in all patients which was most responsible for the discrimination between the two groups.

However, this paper strongly supports the hypothesis that dry-eye disease has an underlying autoimmune mechanism. At this point one cannot decide whether the presence of these autoantibodies is pathogenetic or an epiphenomenon. Considering this autoimmune mechanism, the treatment of dry-eye disease with immunomodulatory drugs such as cyclosporin A can be a very promising approach [20]. The expected beneficial effects of the cyclosporin A treatment on the autoantibody repertoires in tears should thus be analyzed in further studies.

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